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POLYPEPTIDE CLEAVAGES IN THE FORMATION OF POLIOVIRUS PROTEINS*

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The final step in poliovirus morphogenesis appears to be the combination of viral RNA with a protein shell called the procapsid.¹ Concomitant with the union of the RNA and the procapsid there is a cleavage of one of the procapsid proteins, producing two of the four proteins of the virion. We have now found that cleavages play an important role in the formation of most if not all poliovirus-specific proteins. Although most mammalian messenger RNA's appear to be monocistronic, it seems possible that a cleavage mechanism may function in the synthesis of some mammalian cell proteins.

Materials and Methods.—The experimental procedures were similar to those used previously;¹ details are given in the text and figure legends.

DL-ethionine and DL-*p*-fluorophenylalanine were obtained from Sigma Chemical Co.; L-azetidine-2-carboxylic acid, from Calbiochem; and L-canavanine sulfate, from Nutritional Biochemicals Corp.

Results.—Among the major viral proteins² found in the cytoplasm of poliovirus-infected cells, the large polypeptide NCVP 1 is unique in that it is highly labeled only if cells are harvested within about 15 minutes after addition of radioactive amino acids. Relatively little labeled NCVP 1 is present when cytoplasm is examined after labeling for over 30 minutes or after a pulse of label followed by a long chase. Explanations for the disappearance of this large protein include: (a) migration to the nucleus; (b) degradation to small fragments; (c) conversion to other proteins. Hypothesis (a) was excluded by showing that very little poliovirus protein accumulated in nuclei (purified by Penman's method³) and that the spectrum of proteins was similar to that seen in the cytoplasm (M. F. Jacobson, unpublished results). To test hypotheses (b) and (c) the following experiment was performed. Radioactive leucine was added to infected cells for 3 minutes, followed by a 500-fold excess of unlabeled leucine. At short intervals thereafter, aliquots of the cells were harvested and the labeled proteins were fractionated according to molecular weight by electrophoresis on SDS-acrylamide gels. Two representative electropherograms are shown in Figure 1. The preferential disappearance of NCVP 1 is apparent. Its disappearance is mirrored by an accumulation of counts in the region of the gel where the procapsid proteins (VP 0, VP 1, and VP 3) are found, suggesting that NCVP 1 has not been degraded, but has been converted to the procapsid proteins.

If the cleavage of polypeptide NCVP 1 is due to the action of a protease which recognizes a given sequence or three-dimensional configuration of the polypeptide, the cleavage might be prevented by replacement of appropriate amino acids with their analogs. Because *p*-fluorophenylalanine (FPA) is known to inhibit poliovirus growth,⁵ a pulse-chase experiment was carried out using H³-leucine in the presence of FPA. Gel electrophoresis of the proteins synthesized under these conditions (Fig. 2) showed that the breakdown of NCVP 1 had been almost

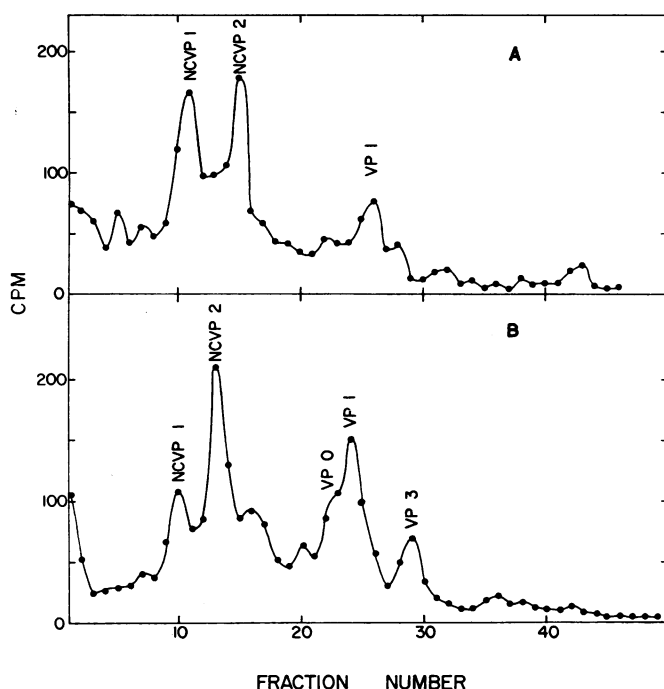


FIG. 1.—Labeling of poliovirus proteins during a pulse and subsequent chase. 8×10^7 infected cells in 20 ml medium were transferred at 3 hr to medium lacking leucine. $300 \mu\text{C}$ of H^3 -leucine were added to the culture for 3 min, followed by a 500-fold excess of nonradioactive leucine. After (A) 2 min or (B) 30 min, 4-ml aliquots of the culture were pipetted into cold Earle's saline. Cytoplasmic extracts were made and analyzed by acrylamide gel electrophoresis as previously described.¹ The anode is at the right in this and subsequent figures, so that the polypeptides are displayed in order of decreasing molecular weight from left to right.

totally prevented and, of the three procapsid proteins VP 0, VP 1, and VP 3, appreciable radioactivity had accumulated only in the VP 1 peak. As discussed below, this peak may not be pure VP 1, but may contain another protein with the same electrophoretic mobility.

FPA also caused appearance of a new protein band between NCVP 1 and NCVP 2 and augmented the relative amount of NCVP 3. In other experiments, using either FPA or FPA plus canavanine, an even larger amount of NCVP 3 accumulated than is shown in Figure 2A.

The success of the first experiments using FPA led us to test a variety of analogs to determine whether additional precursors would accumulate. The most important question was whether the major proteins that accumulated in the presence of FPA were indeed primary gene products or were cleavage products of an even larger polypeptide. Such a polypeptide might never exist as an intact entity under normal conditions if its N-terminal segment (e.g. NCVP 2) were cleaved from a *nascent* protein.

An experiment was performed with four analogs, all of which are known to be

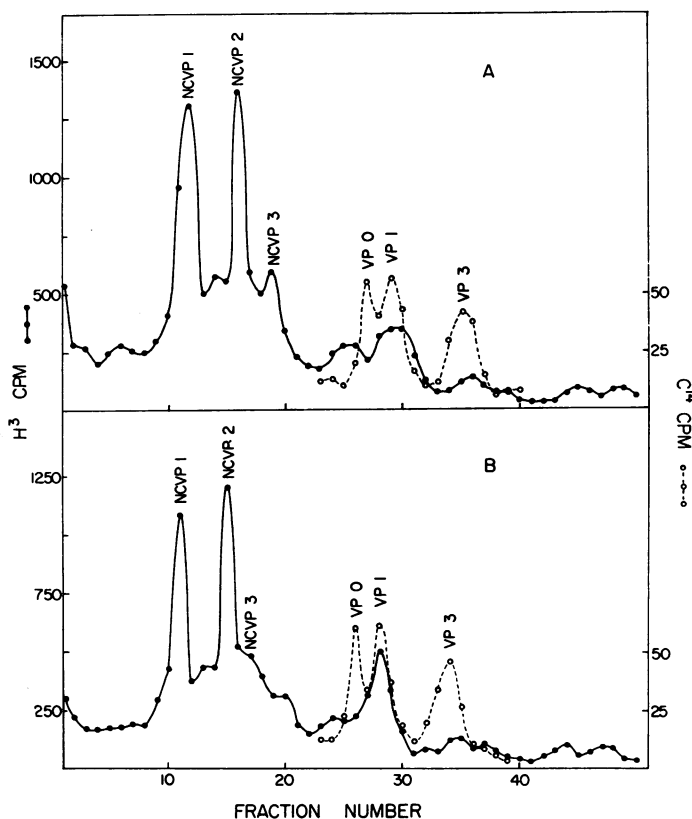


FIG. 2.—Proteins made in the presence of FPA. 4×10^7 HeLa cells in 10 ml medium were infected in the presence of 2 mM guanidine.⁴ At 1 hr 50 min the cells were centrifuged for 2 min at $220 \times g$, washed three times with Earle's saline to remove the guanidine, and resuspended in normal medium containing serum. At 4 hr 30 min cells were centrifuged again, washed twice in Earle's saline to remove amino acids, and resuspended in 10 ml Earle's saline containing 1.1 mM FPA. Cells were incubated for 10 min at 37° and then $130 \mu\text{c}$ of H^3 -leucine were added for 5 min followed by a large excess of unlabeled leucine. Aliquots of cells were harvested (A) 5 min and (B) 45 min later. Cytoplasmic extracts were made and analyzed by gel electrophoresis; C^{14} -procapsid proteins were added as markers. ●—● H^3 ; ○—○ C^{14} .

incorporated into proteins of eucaryotic cells. They were FPA, canavanine⁶ (arginine analog), azetidine-2-carboxylic acid⁷ (proline analog), and ethionine⁸ (methionine analog). The gels used in this experiment were made with 7 per cent acrylamide rather than the usual 10 per cent. This improves the separation of large proteins, but the small proteins are not resolved. As shown in Figure 3A, in the presence of the four analogs, NCVP 1 was formed but no procapsid proteins and relatively little NCVP 2 and NCVP 3 were produced. Moreover, there appeared a new protein, NCVP 0, which is considerably larger than NCVP 1. A small amount of NCVP 0 can also be seen in Figure 2A. Note the almost total absence of radioactivity ahead of NCVP 3. On the plausible assumption that

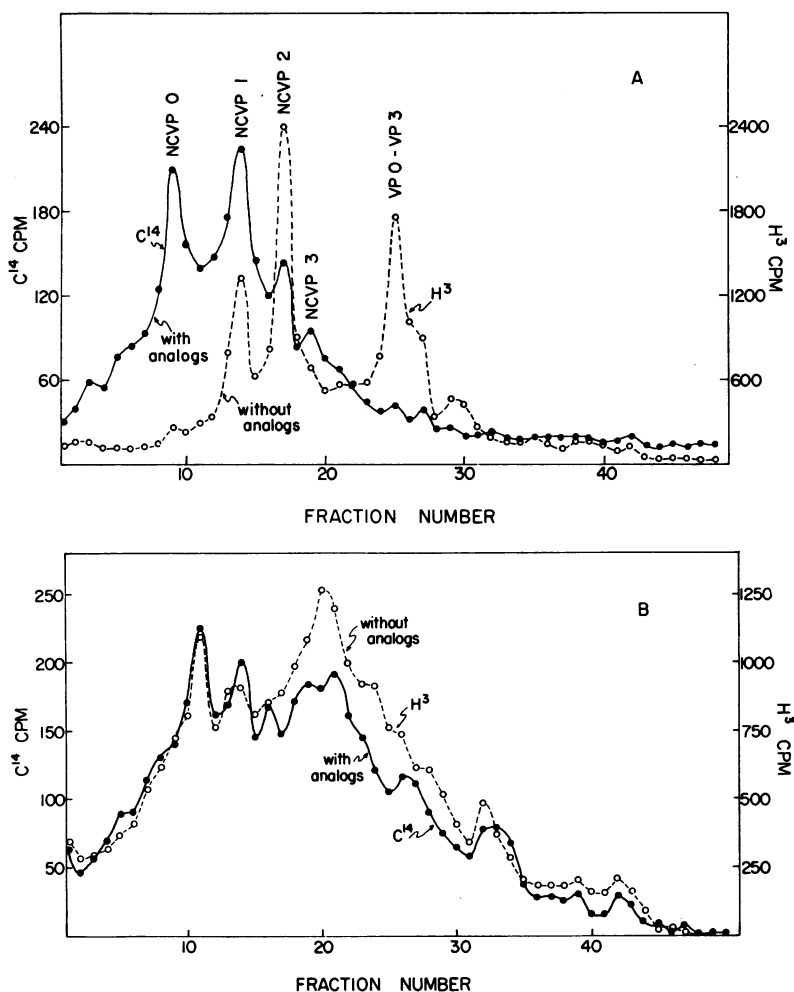


FIG. 3.—Proteins made in the presence of several amino acid analogs. (A) 4×10^7 infected cells in 10 ml medium were centrifuged at 3 hr 30 min and washed twice with Earle's saline. Half the cells were resuspended in Earle's saline, the other half in Earle's saline containing 2.5 mM FPA, 3.3 mM canavanine, 1.8 mM ethionine, and 5.4 mM azetidine-2-carboxylic acid. Both batches were incubated at 37° for 8 min and then 40μ of H^3 -leucine were added to the first half and 10μ of C^{14} -leucine were added to the cells in the presence of analogs. 15 min later unlabeled leucine was added to both, and 2 min after that the cells were harvested. Cytoplasmic extracts were made and analyzed together on a 7% acrylamide gel. (B) An identical experiment was done with uninfected cells. \bigcirc — \bigcirc H^3 -protein made in the absence of analogs; \bullet — \bullet C^{14} -protein made in the presence of analogs.

the amino acid analogs do not interfere selectively with the synthesis of any of the virus-coded polypeptides, this result suggests that none of the proteins smaller than NCVP 3 made under normal conditions is a primary gene product. In addition, some radioactivity was reproducibly present in protein even larger

than NCVP 0, as indicated by the shoulder at fractions 5 and 6 in Figure 3A. In some gels a small peak was seen between NCVP 0 and NCVP 1.

We have assumed that the amino acid analogs cause the appearance of NCVP 0 by becoming incorporated into its structure and preventing its cleavage. It is conceivable, however, that the amino acid analogs might disrupt the protein-synthesizing machinery in such a way that starting and stopping signals encoded in the mRNA are misread, resulting in the synthesis of abnormally large proteins. To test this possibility the proteins of uninfected HeLa cells were labeled in the presence or absence of analogs and analyzed on gels (Fig. 3B). In the presence of high concentrations of several analogs, the size distribution of cell proteins was almost normal; there was, however, a slight shift toward the heavy side. The size of most HeLa cell proteins is therefore unaffected by incorporation of amino acid analogs.

Discussion.—Cleavages of poliovirus proteins: The experiments described above show that none of the proteins of the poliovirus procapsid (VP 0, VP 1, and VP 3) is a primary gene product. This is most clearly demonstrated by the fact that no procapsid proteins are made in the presence of four amino acid analogs. As was suggested previously,¹ the most likely progenitor of the procapsid proteins is NCVP 1; this is supported by the pulse-chase experiment of Figure 1 in which the loss of label from NCVP 1 was paralleled by a large increase of radioactivity in VP 0, VP 1, and VP 3. Summers and Maizel⁹ have also noted this phenomenon. Analyses of tryptic digests of these proteins, now in progress, should provide a definitive test of the relationship among these polypeptides. It should be emphasized that although VP 0, VP 1, and VP 3 are seen as individual species on SDS-acrylamide gels, they may never exist as separate entities, but may remain in an aggregate after cleavage of NCVP 1.

In purified procapsids labeled with radioactive amino acids, approximately equal amounts of label appear in each of the three proteins,¹⁰ whereas when total cytoplasm is analyzed (see Fig. 1B), there is considerably more radioactivity in the VP 1 peak than in VP 0 or VP 3. Furthermore, label appears in the VP 1 peak more rapidly than in VP 0 and VP 3 and, in the presence of FPA (Fig. 2), the VP 1 region is much more heavily labeled than VP 3. These considerations suggest that the VP 1 peak in a gel electropherogram may actually consist of two proteins, one corresponding to VP 1, the other to a hitherto unrecognized protein. This hypothetical protein, which we shall call NCVP X, must also be split off from a larger protein (Fig. 3A). In some experiments with amino acid analogs (Fig. 2) there is a concomitant loss of label in NCVP 3 and increase of label migrating with VP 1, suggesting that NCVP 3 may be the precursor of NCVP X. Experiments are under way to clarify this situation.

Fourteen different virus-specific polypeptides were originally identified in the cytoplasm of poliovirus-infected HeLa cells.⁴ It is now clear that they do not represent fourteen different species independently translated from the genome; rather, at least the major species arise by cleavages of either nascent chains or finished polypeptides. Pulse-chase experiments (Fig. 1; see also refs. 1 and 9) have shown that the polypeptides VP 0–VP 4 are certainly not primary gene products; the use of amino acid analogs indicates that none of the proteins

smaller than NCVP 3 is a primary gene product. Furthermore, under conditions where the newly discovered polypeptide, NCVP 0, appears in large amounts (Fig. 3), the concentrations of NCVP 2 and NCVP 3 are diminished, suggesting that these two polypeptides are derived from NCVP 0, and that only NCVP 0 and NCVP 1 are primary gene products. The existence of some protein considerably larger than NCVP 0 lends credence to the possibility that NCVP 0 and NCVP 1 are themselves split from a larger precursor (NCVP 00) and that poliovirus RNA is in fact translated into a single primary gene product. It is hoped that determining the molecular weights of these large proteins will provide some insight into the relationships among them.

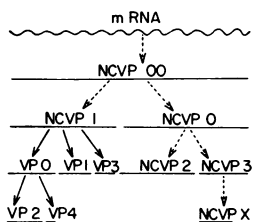


FIG. 4.—Cleavages of poliovirus proteins. It is suggested that the viral RNA directs the synthesis of a single protein (NCVP 00) which is cleaved in several steps to generate the 10–15 viral proteins. Solid arrows indicate cleavages for which the evidence is strong. Broken arrows indicate events for which there is weaker or very little experimental evidence.

As for the other polypeptides synthesized in poliovirus-infected HeLa cells,⁴ none of them is ever found in large amounts. Two of them, NCVP 4 and NCVP 5, may represent transient cleavage states of larger proteins (thus NCVP 1 might first be cleaved into two pieces, one being VP 1, the other consisting of VP 0 plus VP 3). The small polypeptides NCVP 7–NCVP 10 may be either functional proteins or waste fragments created during the cleavage of larger proteins. In the presence of analogs, small peaks are generally seen between NCVP 0 and NCVP 1 and between NCVP 1 and NCVP 2; their derivation is not clear. A summary of the major known and hypothetical cleavage events is shown in Figure 4.

Translation of mammalian messenger RNA: The cleavage of the primary gene product of the poliovirus genome to generate individual proteins represents a distinctly different mechanism from the one used to translate the genome of RNA phages. Although, for both types of viruses, the whole genome is the message,¹¹ phage RNA is directly translated into a number of separate polypeptides.

In this process, which we shall call “internal initiation,” the translation of a single messenger RNA molecule involves termination of polypeptide synthesis and its reinitiation. Probably the best evidence for internal initiation is the finding that at least two of the proteins made *in vitro* from the RNA of phage f2 begin with formylmethionine.^{12, 13} Internal initiation is generally thought also to be the mechanism whereby bacterial polycistronic messages are translated.^{14, 15} Whether mammalian cells ever utilize internal initiation is a question that presupposes the existence of mammalian polycistronic messengers. While such messengers may exist, the available evidence, listed below, favors the hypothesis that most mammalian messenger RNA’s are monocistronic:¹⁶

(1) The polyribosomes that synthesize certain mammalian proteins have been identified. For the two hemoglobin chains,¹⁷ for the two chains of IgG gamma globulin,^{18, 19} and for myosin, tropomyosin, and actin,²⁰ the sizes of the polyribosomes indicate that separate messenger RNA’s are responsible for the synthesis of each polypeptide and that none of these messages is polycistronic.

(2) In certain mouse plasma cell tumors, Kuff and Roberts²¹ found a linear

relationship between the number of ribosomes in a polyribosome and the average length of nascent chain per ribosome. This could not be true if there were significant internal initiation or cleavage of nascent chains (see Kuff and Roberts²¹ for a detailed discussion of this point). Similar analyses for HeLa cells²² and whole chick embryos²³ also showed increasing specific activity with increasing polyribosome size.

In contrast to mammalian polyribosomes, those of bacteria show equal lengths of nascent chains on polyribosomes of all sizes.^{24, 25} This is consistent with the idea that many bacterial messenger RNA's are polycistronic with internal initiation.

(3) For HeLa cells, we determined the lengths of labeled finished polypeptides after a short pulse of labeled leucine and after a long chase (Jacobson and Baltimore, unpublished). No change in the distribution was evident, suggesting that little cleavage of finished chains occurs. Furthermore, the experiment shown in Figure 3B indicates that amino acid analogs do not markedly alter the chain length distribution, so that cleavages of the type blocked by FPA in poliovirus (NCVP 1 \rightarrow VP 0, VP 1, and VP 3) occur rarely, if ever, in uninfected HeLa cells.

The combined evidence of points (2) and (3) above suggests that internal initiation, as well as cleavage of nascent or released polypeptide chains, must not play any major role in mammalian protein synthesis. Most mammalian messenger RNA's, like those few which have been investigated, would therefore be monocistronic. Yet, there are indications that some messages may be polycistronic. In a number of tumors investigated by Kuff and Roberts²¹ the curve of specific activity versus polyribosome size leveled off for very large polyribosomes, an observation which, if not an artifact, may indicate either cleavage of nascent chains or internal initiation. Also, the experiment shown in Figure 3B of the present paper shows that the amino acid analogs do cause a slight shift in the size distribution of HeLa cell proteins. This may be an indication that some cleavage of polypeptide does occur.

The genetic material of other mammalian RNA viruses: It is interesting to compare the structure of the genetic material of three other types of RNA viruses with that of poliovirus. The RNA of reovirus is largely double-stranded and consists of a number of fragments, each of which makes a separate messenger RNA.^{26, 27} Influenza virus contains a number of single-stranded RNA fragments.²⁸ The RNA of Newcastle disease virus is a very long single strand,^{29, 30} but it appears that its messages are short single-stranded regions of RNA complementary to the viral RNA.³¹ These three types of organization of the genome of RNA viruses may have evolved to circumvent the fact that mammalian cells cannot carry out internal initiation. By making it possible to control the times at which different viral genes are expressed, the packaging of genetic information into small units gains for these viruses a flexibility that is lost to poliovirus whose genetic message is translated into a few polypeptides and possibly only one.

Summary.—Pulse-chase experiments with radioactive amino acids indicate that the three procapsid proteins of poliovirus are formed from a single large polypeptide. Incorporation of amino acid analogs into the viral proteins prevents the cleavage of the coat protein precursor as well as the formation of several

other proteins and, furthermore, causes the production of a new polypeptide larger than those previously reported. It is suggested that all the viral proteins may arise by cleavage of a single polypeptide that represents the total information of the viral genome. The possibility is considered that mammalian cells may lack mechanisms for internal initiation of polypeptide synthesis such as occurs during translation of polycistronic messenger RNA in bacteria. The implications of this situation are discussed.

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Note added in proof: After this manuscript was submitted, an article by Holland and Kiehn (these PROCEEDINGS, 60, 1015 (1968)) appeared which demonstrates the occurrence of cleavages during the formation of the proteins of a series of enteroviruses. Recent work in this laboratory (B. Spanier, M. Jacobson, and D. Baltimore) has confirmed their evidence for mengovirus and further shown that FPA blocks the cleavage of a mengovirus polypeptide which is approximately the same size as the poliovirus NCVP 1 (125,000 daltons as judged by its migration rate relative to β -galactosidase, which is 135,000 daltons).

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¹⁶ The term "monocistronic" is used here to describe a messenger RNA producing a single polypeptide which is not cleaved to produce multiple functional polypeptides. "Polycistronic" denotes a messenger RNA which ultimately yields more than one functional polypeptide (either by cleavage or internal initiation).

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